Activity of bone morphogenetic protein-7 after treatment at various temperatures: Freezing vs. pasteurization vs. allograft

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Munetomo Takata, M.D.[1], Naotoshi Sugimoto, Ph.D.[2], Norio Yamamoto, Ph.D.[1], Toshiharu Shirai, Ph.D.[1], Katsuhiro Hayashi, Ph.D.[1], Hideji Nishida, Ph.D.[1], Yoshikazu Tanzawa, Ph.D.[1], Hiroaki Kimura, Ph.D.[1], Shinji Miwa, M.D.[1], Akihiko Takeuchi, Ph.D.[1], Hiroyuki Tsuchiya, Ph.D.[1]

[1]Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan

[2]Department of Physiology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan

Corresponding author: Hiroyuki Tsuchiya, Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan; Tel: +81-76-265-2374; Fax: +81-76-234-4261; E-mail address: tsuchi@med.kanazawa-u.ac.jp

Abstract

Insufficient bone union is the occasional complication of biomechanical reconstruction after malignant bone tumor resection using temperature treated tumor bearing bone; freezing, pasteurization, and autoclaving. Since bone morphogenetic protein (BMP) plays an important role in bone formation, we assessed amount and activity of BMP preserved after several temperature treatments, including -196 °C and -73 °C for 20 min, 60 °C and 100 °C for 30 min, 60 °C for 10 h following -80 °C for 12 h as an allograft model, and 4 °C as the control. The material extracted from the human femoral bone was treated, and the amount of BMP-7 was analyzed using an enzyme-linked immunosorbent assay. Then, the activity of recombinant human BMP-7 after the treatment was assessed using a bioassay with NIH3T3 cells and immunoblotting analysis to measure the amount of phospho-Smad, one of the signaling substrates that reflect the intracellular reaction of BMPs. Both experiments revealed that BMP-7 was significantly better preserved in the hypothermia groups. The percentages of the amount of BMP-7 in which the control group was set at 100% were 114%, 108%, 70%, 49%, and 53% in the -196 °C, -73 °C, 60 °C, 100 °C, and the allograft-model group, respectively. The percentages of the amount of phospho-Smad were 89%, 87%, 24%, 4.9%, and 14% in the -196 °C, -73 °C, 60 °C, 100 °C, and the allograft-model group, respectively. These results suggested that freezing possibly preserves osteoinductive ability than hyperthermia treatment.

Key words.

Bone tumor; freezing; bone morphogenetic protein; protein stability; allograft; liquid nitrogen; cryotherapy; osteoinductive ability

Introduction

Massive bone loss after tumor resection requires various types of reconstruction. Surgeons have multiple alternatives, including massive endoprosthesis, allograft, composite arthroplasty, and distraction osteogenesis [20]. The allograft is the most frequently used substitute, but recently, recycled resected tumor-bearing bones have been increasingly used. Treatment methods include irradiation [1], autoclaving [2], pasteurization [13,18], and freezing using liquid nitrogen [21,22]. These methods offer significant advantages and overcome a need for a bone bank system, which is necessary for allografts and not preferable in some Asian countries for socio-religious reasons. Since procedures that enable both elimination of all tumor cells and re-implantation of the recycled bone in the patient needs to be completed within a short time during operation, hyperthermia or hypothermia treatments are useful because they are simple and less time-consuming.

There are several methods to treat tumor-bearing bones with temperature. Tumor bones are treated at 135 °C for 10 min in an autoclave. During this procedure, the osteoinductive property, which is defined by the proteins inside the treated bone, apparently declines. Pasteurization is superior to autoclaving in retaining bone inductivity with lower hyperthermia treatment at 60–65 °C for 30–40 min [13]. Like the heating processes, which can devitalize tumor cells, freezing exerts a similar effect by inducing ice crystal formation

and dehydration of the cells. Only one cycle of -196 °C for 20 min is sufficient to kill all tumor cells [25]. This newly developed method using frozen autografts has become popular since we reported it for the first time in 2005. Cold conditions are also used for the long-term preservation of the freshness of tissues, such as allografts. In general, they are stored preferably below -70 °C. However, immediately before using allograft bones, heat treatment at 60 °C for 10 h is necessary to avoid the transmission of infectious viruses [11].

In the clinical aspect, common complications such as fracture, nonunion, and delayed union occur at various rates (14% [21], 20% [13], and 22% [2], respectively), and iliac crest bone grafting could sometimes be required after reconstruction using recycled bones. The cause of insufficient bone formation is supposed to be complicated: wide resection of soft and bone tissues around the tumor and death of normal osteocytes and osteoblasts. In addition, damage to the proteins such as bone morphogenetic protein (BMP) induced by the sudden change in temperature could be a reason. BMPs are osteoinductive agents and induce differentiation of mesenchymal-type perivascular cells into cartilage and bone. BMP-7, also known as osteogenic protein 1 (OP-1), is a member of the transforming growth factor β (TGF-β) superfamily of proteins and is able to induce new bone formation *in vivo* [17].

Although an experimental study showed some amount of preservation of BMP during pasteurization [14], there have been no studies comparing the efficacies of each temperature treatment method directly. In this study, we attempted to compare the extent of damage to

BMP-7 after treatment at various temperatures.

Materials and Methods

This research was approved by the institutional review board.

We performed two experiments. First, we assessed the amount of the BMP-7 extracted from human femoral bone head by using an enzyme linked immunosorbent assay (ELISA). Next, we assessed the activity of recombinant human BMP-7 (rhBMP-7) using NIH3T3 mouse fibroblast cells.

In both, we divided the samples into 6 groups, in which each temperature treatment was the same. First, we defined 4 groups of treatment, i.e., -196 °C for 20 min, 60 °C for 30 min, and 60 °C for 10 h following -80 °C for 12 h which represented an allograft model. Samples stored at 4 °C in a refrigerator served as control. The following technical equipment was used: a water bath (Thermominder SD mini; TAITEC Inc., Koshigaya, Saitama, Japan), a high temperature chamber (ETAC Junior SA01; Kusumoto Chemicals Inc., Kanda, Tokyo, Japan), and an ultralow-temperature freezer (MDF-U383; Sanyo Inc., Moriguchi, Osaka, Japan). Each temperature and the treatment time were defined on the basis of clinical usage [13,21,23]. Next, we added 2 groups to assess the further influence of hypo/hyperthermia

treatment: -73 °C created from the combination of treatment with dry ice and liquid ethanol for 20 min and 100 °C treatment in boiling water for 30 min. Dry ice which is the solid state of carbon dioxide has its boiling point at -79 °C, and the melting point of liquid ethanol is -117 °C. Simple combination of those 2 popular materials results in a liquid of high hypothermia that can freeze samples immediately, similar to liquid nitrogen.

All samples were kept in the refrigerator at 4 °C except while they are treated.

Human sample purification

A fresh human femoral bone head was excised during hemiarthroplasty. The consent obtained from donors included use for research. The head and the reamed bone weighed 92 g in total and were comminuted into small pieces with a bone grinder. Then, 80 ml of phosphate-buffered saline (PBS) was added and the turbid liquid was divided into three 50-ml tubes, stirred sufficiently, and centrifuged at 1500 rpm for 5 min. After centrifugation, three layers were obtained: a yellow and clear fat layer at the top, a light-red layer in the middle, and a bone tip layer at the bottom. The middle layer was removed as sample. We obtained total amount of 50 ml and delivered it into 500-μl micro tubes. All samples were kept in the refrigerator at 4 °C immediately after the extracted, and all the temperature-treatments and ELISA analysis were processed within 1 day.

Chemicals

RhBMP-7 was obtained from R&D SYSTEMS Inc. (Minneapolis, MN). It is derived from Chinese hamster ovary cell line. The structure of the rhBMP is disulfide linked homodimer. The product had been reduced by sodium dodecyl sulfate polyacrylamide gel electrophoresis, so it was reconstituted at 100 μg/mL in sterile 4 mM HCl, just before the usage. Anti-phospho-Smad1/5 (Ser463/465) (41D10), anti-Smad1, and horseradish peroxidase (HRP)-linked anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

ELISA analysis

BMP-7 from human femoral bone head was analyzed with an ELISA Kit (RayBioteck Inc, Norcross, GA). We added 100 μl of each of the treated samples into each well. The kit contains 96 well-plates, and all 24 samples were run in duplicate. We measured the absorbance on a microplate reader at 450 nm and calculated the concentration of BMP-7 according to the standard curves plotted on log-log graph paper.

Cell culture

NIH3T3 (NIH3T3/14-1) mouse fibroblast cells were obtained from Riken BioResource Center (Ibaragi, Japan) and maintained in Dulbecco's modified eagle medium (DMEM)

supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator.

Bioassay and immunoblotting analysis

BMP signals are mediated by type I and II BMP receptors which form a signal-transducing complex. Once BMP-7 binds to the extracellular matrix of the receptor, Smad1, 5, and 8, the signaling substrate, become phosphorylated. Phosphorylated Smad1 or 5 interacts with Smad4 and enters the nucleus to activate the transcriptional machinery for early BMP-response genes [17]. To evaluate the intracellular activity of BMP-7 in cells that were treated at several temperatures, we measured the amount of Smad1 and phosphorylated Smad1 using a Smad1 and Phospho-Smad1/5 antibody in immunoblotting.

NIH3T3 cells were incubated in DMEM without serum for 12 h and treated with rhBMP-7 for 10 min. Cell extracts were immunoblotted using anti-phospho-Smad1/5 or anti-Smad1 antibodies. Immunoblotting was performed as described previously [19]. Briefly, proteins were extracted from cells, and the protein concentrations were determined using the protein assay reagent. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with the first antibody (1:1000), followed by incubation with the respective HRP-linked secondary antibody (1:2000). The blots were developed using the Immobilon Western

Chemiluminescence HRP Substrate (Millipore, Billerica, MA).

We also made the standard line according to the various concentrations of the rhBMP-7 which are not treated by temperature.

Statistical analysis

Data are presented as means (SEM) of at least 3 independent experiments. Statistical analysis was performed by ANOVA followed by Dunnett's test.

Results

ELISA analysis

BMP-7 was present in all samples after treatment. The mean concentration in each group was 164 (3.16) pg/ml in the 4 °C group, 187 (8.2) pg/ml in the -196 °C group, 177 (8.3) pg/ml in the -73 °C group, 114 (13.3) pg/ml in the 60 °C group, 80 (9.19) pg/ml in the 100 °C group, and 87 (18.6) pg/ml in the allograft group. The SD ranged from 3.16 to 18.6 which reflected the small variation in each group. The results were calculated as % appearance, in which the value of the control group (164 pg/ml) was set 100% (Fig. 1). The calculated percentages were 114% in the -196 °C group, 108% in the -73 °C group, 70% in the 60 °C group, 49% in the 100 °C group, and 53% in the allograft group (SD 18.6). In both hypothermia groups, i.e.,

-196 °C and -73 °C, BMP-7 was preserved equally as in the control group, and there were significant differences between the hypothermia and hyperthermia groups. In the hyperthermia groups, the amount of BMP-7 was significantly decreased depending on the temperature and treatment duration, and there was no significant difference between the 100 °C group and the allograft group.

Effect of temperature on BMP-7 bioactivity

Because the total amount of Smad1 in each sample was equally detected, the background of the cells was assumed to be equivalent to each other. However, there was a clear difference in the appearance of the phospho-Smad band in each group (Fig. 2). The densities of the bands were compared and that of the control group (4 °C) was standardized to 100% (Fig. 3). The mean density of each group was 89 (1.4)% in the -196 °C group, 87 (3.5)% in the -73 °C group, 24 (2.8)% in the 60 °C group, 4.9 (1.8)% in the 100 °C group, and 14 (3.3)% in the allograft group. In the hypothermia groups, the phospho-Smad band was detected as thick as that of the control group without significant difference; though, the density was slightly decreased. In the hyperthermia groups, the concentration of phospho-Smad was significantly decreased depending on the temperature and treatment duration, and the lowest concentration was noted in the 100 °C group.

According to the standard line from Fig. 4, the concentration in each group was: 463 ng/ml in

the 4 °C group, 231 ng/ml in the -196 °C group, 196 ng/ml in the -73 °C group, 3.19 ng/ml in the 60 °C group, 0.888 ng/ml in the 100 °C group, and 1.60 ng/ml in the allograft group.

Discussion

This is the first time that the change in BMP amount and activity after treatment with hyper- and hypothermia has been compared experimentally. The results of ELISA analyses and bioassays were correlated and revealed that BMP-7 was best preserved in hypothermia-treated groups. Among the hyperthermia groups, BMP-7 was better preserved in the 60 °C group, but there was a significant decrease compared to the hypothermia groups. The main reason of denaturation in the allograft group was supposed to be the step of hyperthermia treatment at 60 °C for 10 h. The 100 °C group showed the greatest induction of denaturation.

Between the -196 °C and -73 °C groups, there was no significant difference in amount and activity of BMP. During nearly all the duration of hypothermia treatment, samples were in the solid state, in which hardly any degeneration occurred. On the other hand, in the hyperthermia groups, significant differences in amount and activity of BMP were seen depending on the temperature and treatment duration.

In the ELISA experiments, a slight increase in BMP-7 was seen in the hypothermia groups

compared to the control group. Other proteins, e.g., proteases, seemed also to be affected to the control group [14], but there had no detailed report on that.

Although there are experimental studies showing some amount of preservation of BMP after hyperthermia treatment [14,16], they did not include comparison with hypothermia treatment. Nakanishi et al. reported preservation of BMP in heat-treated bones [14]. BMP extracted from rabbit bones after heat treatment for various times at different temperatures (from 50 °C to 125 °C) was implanted into the muscles of mice to evaluate its osteogenetic activity. Maximum new bone formation was observed in specimens treated at 70 °C for 10 min. They supposed that the interaction of BMP and BMP-ase was the main reason why BMP extracted from heat treated bone at 50 °C or 60 °C failed to induce bone formation. Ohta et al. reported that the biological activity and molecular structure of rhBMP-2 was preserved after lower hyperthermia treatment of 50 °C and 70 °C compared to the higher hyperthermia treatment of 90 °C and 100 °C [16]. These results support the present study in which the activity of BMP was decreased depending on the temperature and treatment duration in the hyperthermia groups.

Preservation in the hypothermia-state is popular in daily life, especially for foods, including vegetables, fish, meat, milk, and so on. In the field of medical science, many types of cells, tissues, and other biomaterials are also preserved in the cold state, using sophisticated protocols [10]. However, there are several reports on cold denaturation [3,4,5,7]. The

suggested mechanism is dehydration due to growing ice crystal formation which results in an increase in salt concentration. In addition, by lowering the pH, proteins can destabilize and precipitate [5]. Loss of the hydrophobic effect that plays an important role in stabilizing proteins is also thought to be attributed to denaturation [7]. However, it is not possible yet to assess the dynamic state of the protein structure along with the change in temperature; although, several techniques exist, including X-ray crystallography, nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy, etc [5]. The structure of BMP-7 has already been determined by X-ray crystallography and is similar to that of other TGF-β superfamily proteins [9]. Its monomer fold consists of 1 α-helix, 8 β-sheets, and 3 disulfide bonds, which can form stable complexes [8]. It is possible that this structure also contributes to the tolerance for the change in temperature.

The exact amount of BMP inside each bone has not been determined yet. With the use of dissociative extractants such as 4 M guanidine hydrochloride, an estimated amount of 2 to 3% of BMP could be extracted [17]. The amount of BMP-7 required for the formation of new bones also has not been determined yet. The commercially available BMP, OP-1 (Stryker Inc. Hopkinton, MA), contains 3.5 mg BMP-7 in 1 unit. By using compressive strains, the amount of BMP-7 released from the fresh-frozen femoral head allograft has been demonstrated to be approximately 4 times as high at 80% strain compared to at 20% strain [6]. Thus, more BMP-7 could be obtained depending on the impaction procedure.

Limitation of this study is the different state of the treatment from the clinical usage. It is unclear whether the BMPs are more damaged when contained in the en bloc bone or in solution. In experiments, BMP-7 in solutions can be easily used because of its uniform concentration; whereas, the BMP amount could differ inside an en bloc bone.

In addition, hypothermia treatment has the potential to preserve other proteins. Cryotreatment to the tumor-bearing bone induces anti-tumor effects [12,15]. Hypothermia induces tumor cell death as well as may activate a systemic anti-tumor immune response stimulated by tumor antigens released by cryonecrotic tissues [24]. In this aspect, preservation of the tumor antigens that determines the level of immune response is also important in clinical settings.

In conclusion, freezing of tumor-bearing bones possibly results in better osteoinductive ability than hyperthermia treatment.

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Figure legends:

Figure 1: Enzyme-linked immunosorbent assay analysis

Each concentration was calculated as % appearance, in which the 4 °C group was standardized as 100%. In both hypothermia groups, bone morphogenetic protein (BMP)-7 was preserved equally as in the control group. There was a significant difference between the hypo- and hyperthermia groups.

Temperature treatment: The 4 °C group was in a refrigerator and served as control, the -196 °C group was in liquid nitrogen for 20 min, the -73 °C group was treated with the combination of dry ice and liquid ethanol for 20 min, the 60 °C group was in a water bath for 30 min, the 100 °C group was in boiling water for 30 min, and the allograft model group was in a high temperature chamber set as 60 °C for 10 h following storage in a freezer set at -80 °C for 12 h.

Figure 2: Band appearance after immunoblotting

Smad-1 was equally detected in each group. The thickness of the phospho-Smad band reflects the activity of bone morphogenetic protein (BMP)-7. The bands of the hypothermia groups were as thick as that of the 4 °C control group.

Figure 3: Amount of Phospho-Smad1/5

The thickness of each band was calculated as % appearance, in which the 4 °C group was set as standard. In both hypothermia groups, bone morphogenetic protein (BMP)-7 was preserved equally as in the control group. There was a significant difference between the hypo- and hyperthermia groups.

Figure 4: Standard line for immunoblotting

Each data point was plotted on a semi-log graph. The supposed straight line from the plots corresponds to the logarithmic function of $Y = 0.350\log \times -0.067$.

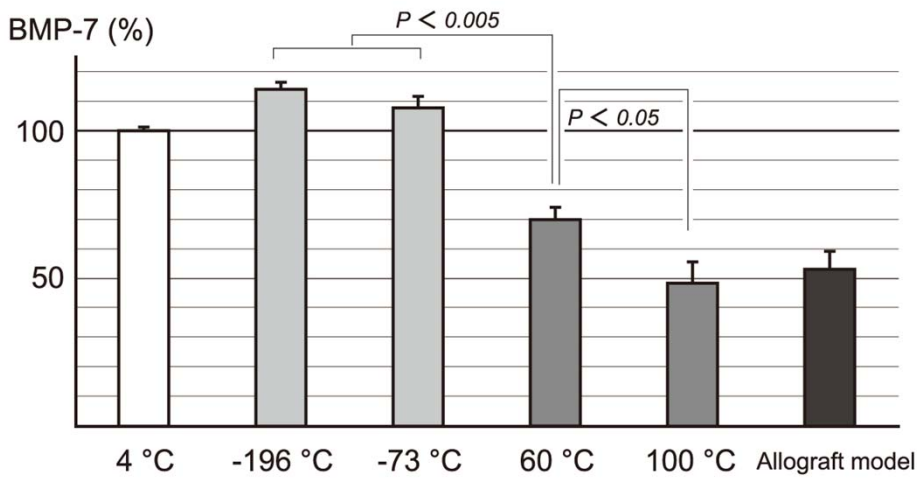


Figure 1: ELISA analysis

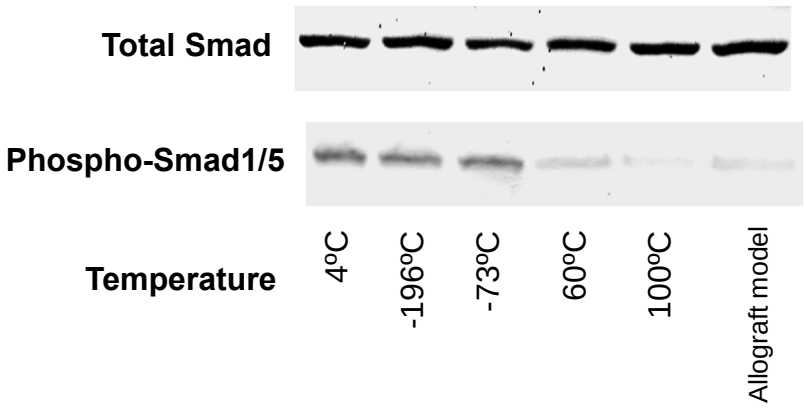
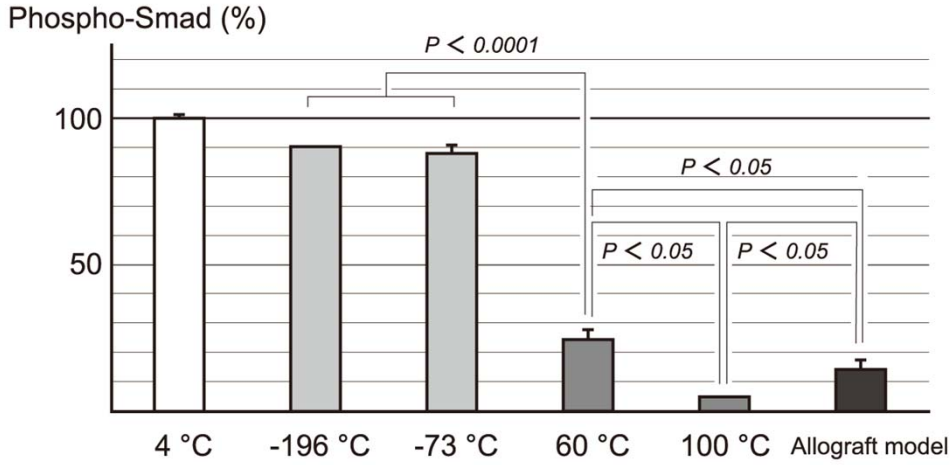


Figure 2: Band appearance after immunoblotting



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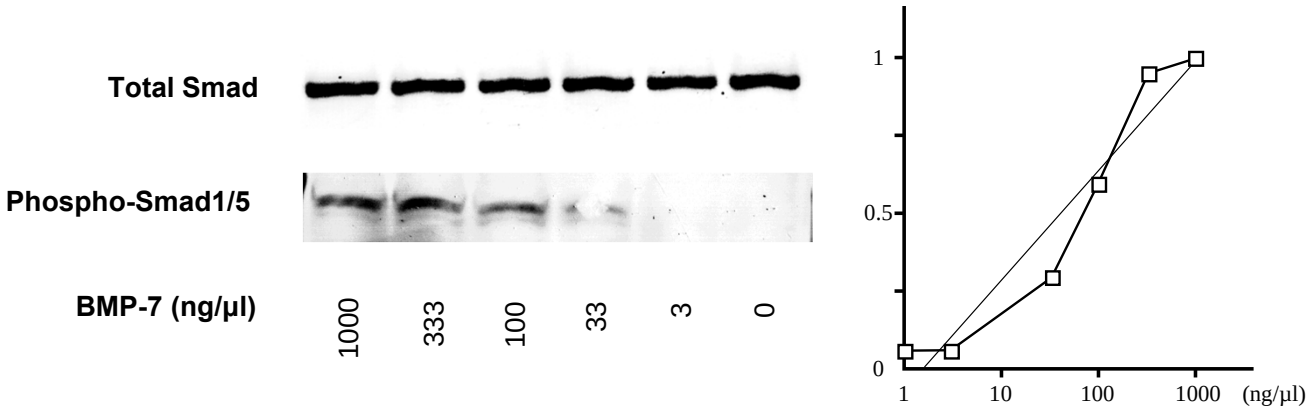

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